v-Ras and protein kinase C dedifferentiate thyroid cells by down-regulating nuclear cAMP-dependent protein kinase A

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Ras proteins are membrane-associated transducers of external stimuli to unknown intracellular targets. The constitutively activated v-ras oncogene induces dedifferentiation in thyroid cells. v-Ras appears to act by stimulating protein kinase C (PKC), which inhibits the nuclear migration of the catalytic subunit of the cAMP-dependent protein kinase A (PKA). Nuclear tissue-specific and housekeeping *trans*-acting factors that are dependent on phosphorylation by PKA are thus inactivated. Exclusion of the PKA subunit from the nucleus could represent a general mechanism for the pleiotropic effects of Ras and PKC on cellular growth and differentiation.

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The *ras* genes encode membrane-associated proteins that bind guanine nucleotide and transduce environmental signals to unknown intracellular targets (see Hall 1990; Bourne et al. 1991). Oncogenic *ras* derivatives promote transformation and cellular proliferation. They can also affect cellular differentiation (Beug et al. 1982; Freytag 1988). In myoblasts, v-Ras blocks maturation by inhibiting the synthesis and activity of MyoD, a nuclear *trans*-acting factor that induces terminal differentiation (Lasser et al. 1989).

Our studies have utilized the TL (FRTL-5) rat thyroid cell line. TL cells are differentiated and express at least three thyroid-specific markers: thyroglobulin, thyrotropin-stimulated hormone (TSH) receptor, and a sodium-iodide cotransporter (Ambesi-Impiobato et al. 1980). The transcription of the thyroglobulin gene is known to be associated with the presence of the homeo box *trans*-acting factor TTF1 (also termed TgTf1). TTF1 binds to the -60-nucleotide region of the thyroglobulin promoter (pTg) (Musti et al. 1987; Ursini et al. 1989; Guazzi et al. 1990; Avvedimento et al. 1991). Our data suggest that the DNA-binding activity of TTF1 is dependent on phosphorylation by cAMP-dependent protein kinase A (PKA) (Avvedimento et al. 1991).

To examine the mechanism by which v-Ras induces dedifferentiation, TL cells were transformed with a Kirsten

murine sarcoma virus (Ki-MSV) variant that expresses a temperature-sensitive v-Ras protein. The transformed line (Ats) did not express differentiation markers or a pTg-NEO fusion (Colletta et al. 1983). Treatment of Ats cells with 5-azacytidine and selection for G418 resistance yielded a derivative clone (Ats-aza) that transcribed the thyroglobulin and thyrotropic receptor genes at 39°C, the nonpermissive temperature for v-Ras. Ats-aza cells exposed to active v-Ras by shift down to 33°C rapidly lost the ability to express thyroid-specific markers (Avvedimento et al. 1989).

We have shown that v-Ras provokes dedifferentiation in TL cells by down-regulating TTF1. Nuclear extracts isolated from cells exposed to v-Ras lacked TTF1 but could be reactivated by incubation of the extracts with PKA. Therefore, we proposed that v-Ras inhibited TTF1 phosphorylation by PKA. Consistent with this hypothesis, we found a twofold reduction in PKA activity in cells exposed to v-Ras (Avvedimento et al. 1991).

A direct causal linkage between v-Ras and lowered PKA activity appears unlikely. Although Ras is a G protein and acts as a positive effector of adenylyl cyclase in *Saccharomyces cerevisiae*, no evident linkage between the two functions has been demonstrated in higher eukaryotes (Toda et al. 1986). Thyroid cells exposed to v-Ras could not be induced to redifferentiate with cAMP derivatives (Avvedimento et al. 1991).

Therefore, we chose to search for a possible mediator between v-Ras and PKA. A reasonable candidate for such

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a mediator is protein kinase C (PKC). In several cell lines, an antagonistic relationship exists between PKA and PKC (Mechta et al. 1989). Furthermore, v-Ras is known to activate PKC, and PKC potentiates transformation by v-Ras (Lacal et al. 1987; Hsiao et al. 1989). The evidence presented in this paper suggests that v-Ras does act through PKC to down-regulate PKA, apparently by excluding the PKA catalytic subunit from the nucleus.

Results

PKC mimics v-Ras

Figure 1 shows that stimulation of PKC with TPA induced dedifferentiation in Ats-aza cells. Ats-aza cells grown at 39°C were treated with TPA (12 ng/ml), and nuclear extracts were prepared for gel shift-binding assays, by using an oligonucleotide [oligo(C)] derived from the -60-nucleotide region of pTg. Figure 1A shows a marked reduction in TTF1 activity after 3–6 days of culture in TPA (Fig. 1A, lanes 1–3). Cells treated for 1 week with TPA had undetectable TTF1 levels, comparable to the transformed Ats parental line (Fig. 1A, lanes 4,5). As

Figure 1. (A) PKC stimulation inhibits TTF1 binding. TTF1 binding to oligo(C) was assayed by gel retardation as described in Materials and methods. Nuclear extracts were prepared from TL or Ats-aza cells incubated at 39°C (lanes 1,2), Ats-aza cells treated with TPA (12 ng/ml) for 3 days (lane 3) or 1 week (lane 4), or undifferentiated Ats cells at 39°C (lane 5). The specificity of the retarded complexes was established by competition with nonspecific oligonucleotides and with an oligonucleotide mutated in the core-binding region. (B) Complex A in TPA-treated cells. The extracts described above were analyzed for binding to oligo(A). (C) Reactivation of TTF1 after TPA removal does not require de novo protein synthesis. TTF1 binding in Ats-aza cells (lane 1) was treated with TPA (12 ng/ml) for 1 week (lane 2). TPA was removed, and the cells were further incubated at 39°C for 1 day in the presence (lane 3) or absence (lane 4) of cycloheximide (10 μ g/ml). Cycloheximide inhibited 90% of [35 S]methionine incorporation (data not shown).

Figure 2. PKC stimulation reduces pTg-NEO mRNA levels. Shown is a Northern blot analysis of RNA from Ats-aza cells (lane 1) treated with TPA (12 ng/ml) for 1 week (lane 2). Hybridization was to the *Bgl*II-*Nco*I fragment of the neomycin resistance gene (upper arrow). The same blot was hybridized with an actin probe (lower arrow). Normalized to the actin RNA levels, the amount of pTg-NEO in TPA-treated Ats-aza cells was five- to sevenfold lower than in untreated controls.

control, we measured the levels of factor A in the same nuclear extracts. Factor A is a non-thyroid-specific nuclear *trans*-acting factor that binds an oligonucleotide [oligo(A)] derived from the -150 region of pTg. The activity of factor A was not significantly affected by TPA (Fig. 1B). Ats-aza cells cultured with TPA for 1 week regained full TTF1 binding within 24 hr after removal of the drug. TTF1 reactivation was resistant to cycloheximide (Fig. 1C). The kinetics of TTF1 loss induced by TPA, the rapidity of recovery upon TPA removal, and the resistance of TTF1 reactivation to cycloheximide were identical to the effects of transient exposure of Ats-aza cells to v-Ras by temperature shift (Avvedimento et al. 1991).

The loss of TTF1 activity in TPA-treated cells was associated with a decrease in the abundance of mRNA expressed from the thyroglobulin promoter. The levels of pTg-NEO mRNA were reduced >80% by treatment of Ats-aza cells with TPA for 1 week (Fig. 2). The linkage between TTF1 levels and pTg activity was observed previously in Ats-aza cells exposed to v-Ras (Avvedimento et al. 1989) or in TL cells starved for thyrotropin (A.M. Musti, in prep.).

Sphingosine induces differentiation

The parallels between the effects of TPA and v-Ras suggest that v-Ras might require active PKC to induce dedifferentiation. In the following experiment, we demonstrate that inhibition of PKC suppresses v-Ras (Fig. 3). Ats-aza cells were grown for 1 week at 33°C to activate v-Ras and were then treated with sphingosine, a potent inhibitor of PKC (Hannun et al. 1986; Hidaka and Hagiwara 1987; Hall et al. 1988). After 24 hr of treatment with sphingosine, we observed a small but significant reactivation of TTF1 (Fig. 3, lanes 2,3). The addition of 1 mM 8-bromo-cAMP (8-Br-cAMP) to the medium, which had little effect on TTF1 activity by itself, potentiated

Figure 3. Inhibition of PKC restores TTF1 activity in v-Ras-exposed cells. TTF1 binding in nuclear extracts of Ats-aza cells in the absence (39°C) or presence (33°C) of active v-Ras (lanes 1,2). Ats-aza cells grown at 33°C for 1 week were treated for 2 days with 10 μ M sphingosine (lane 3), 1 mM 8-Br-cAMP (lane 4), or both (lane 5).

the action of sphingosine (Fig. 3, lanes 4,5). By averaging the results of several experiments (Table 1), we calculate that nuclear extracts from Ats-aza cells shifted to 33°C for 1 week had 2% of the initial TTF1 activity. Treatment of these cells with 8-Br-cAMP or sphingosine raised TTF1 activity to 6% and 20% of the 39°C levels, respectively. Cells treated with both sphingosine and 8-Br-cAMP, however, reached 65% of the initial TTF1 activity. Depletion of PKC by treatment with high TPA

(220 ng/ml for 12 hr) reproduced the effects of sphingosine on TTF1 in Ats-aza cells cultivated at 33°C (Fig. 4).

Reactivation of TTF1 by sphingosine or by sphingosine and 8-Br-cAMP coincided with increased pTg-NEO mRNA levels (Fig. 5).

Treatment of the v-Ras-exposed Ats-aza cells with sphingosine and 8-Br-cAMP, alone or together, neither stimulated nor inhibited factor A binding to oligo(A) (data not shown).

PKC inhibits PKA-dependent trans-acting factors

TTF1 binding is destroyed when nuclear extracts are incubated with acid phosphatases and can be reactivated by phosphorylation of the treated extracts with PKA (Avvedimento et al. 1991). In Figure 6 we show that TTF1 activity in Ats-aza cells incubated with sphingosine and 8-Br-cAMP was likewise sensitive to phosphatase (Fig. 6, lanes 2,3). Phosphorylation of the phosphatase-treated extracts with the PKA catalytic subunit restored TTF1 binding (lane 5). Incubation with PKC of nuclear extracts from differentiated TL or Ats-aza cells had no effect on TTF1 binding and neither reactivated phosphatase-treated TTF1 nor inhibited phosphorylation by PKA (lanes 4,6).

These results support the idea that v-Ras acts through PKC to down-regulate PKA. The low activity of PKA is, in part, also the result of reduced cAMP levels, because the effects of sphingosine are potentiated by 8-Br-cAMP. Adenylate cyclase activity is reduced in cells trans-

Figure 4. High phorbol ester (TPA) concentrations reactivate TTF1 in v-Ras-exposed cells. Nuclear TTF1 activity was determined by gel retardation assay. (Lane 1) Ats-aza cells at 39°C; (lane 2) Ats-aza cells grown at 33°C for 2 weeks; (lane 3) as in lane 2 and then treated with TPA (230 ng/ml) for 48 hr followed by treatment with forskolin (50 μ g/ml) for 15 hr. Inhibition of cytosolic PKC was followed by histone H3 phosphorylation in the presence or absence of specific phospholipids (Avvedimento et al. 1991). Cytosolic phospholipid-stimulated kinase activity was fivefold higher in cells cultured at 33°C than at 39°C, in agreement with PKC assays of wild-type v-Ras-transformed thyroid cells (Spina et al. 1988). Depletion of cytosolic PKC was maximal after 24–48 hr of treatment with TPA at 230 ng/ml; longer treatment was less effective (data not shown).

Table 1. PKC inhibits TTF1 and CREB

Temperature (°C)	Treatment	TTF1 (%)	CREB (%)
39	none	100	100
33	none	2 $\pm$ 2	4 $\pm$ 3
33	cAMP	6 $\pm$ 5	25 $\pm$ 8
33	sphingosine	20 $\pm$ 6	70 $\pm$ 20
33	cAMP + sphingosine	65 $\pm$ 20	75 $\pm$ 20
39	TPA (10 ng/ml)	10 $\pm$ 4	15 $\pm$ 8
33	TPA (220 ng/ml)	75 $\pm$ 20	80 $\pm$ 24

The treatment of Ats-aza cells and the band shift analysis of nuclear extracts are described in the legends to Figs. 3 and 7. TTF1 and CREB efficiency was quantified by excising the retarded oligonucleotide band [oligo(C) and CRE, respectively] from the acrylamide gel and determining its radioactivity in a liquid scintillation counter. The results represent an average of at least three experiments and are presented as percent of the value found in Ats-aza cells at 39°C.

Figure 5. Sphingosine and cAMP treatment restores pTg-NEO mRNA levels in cells exposed to v-Ras. Shown is a Northern analysis of total RNA from Ats-aza cells cultured at 39°C (lane 1), shifted to 33°C for weeks (lane 2), and treated with 10 μ M sphingosine and 1 mM 8-Br-cAMP (lane 3). (Top) RNA hybridizing with a specific neomycin-resistance gene probe (see legend to Fig. 1). The arrow indicates pTg-NEO mRNA. (Bottom) The band in lane 1 is an artifact resulting from nonspecific competition of 18S RNA with pTg-NEO mRNA. The same blot was hybridized with rat GAPDH cDNA as control [arrow].

formed with v-Ras owing to the down-regulation of the TSH receptor (Berlingieri et al. 1990).

We have also assayed a second cAMP-dependent DNA-binding factor in TPA-treated cells (Grove et al. 1987; Montminy and Belezikjan 1987; Day et al. 1989; Merlino et al. 1989). Nuclear extracts from Ats-aza cells grown at 39°C formed a retarded DNA-protein complex with an oligonucleotide containing the cAMP responsive element (CRE) of a mouse *c-fos* promoter (Fig. 7A, lane 2). Extracts from TPA-treated cells formed significantly less complex (Fig. 7A, lanes 3–5). Likewise, cells cultured at 33°C had little CRE-binding activity; binding was restored by treatment with sphingosine and 8-Br-cAMP (Fig. 7B). Incubation of these cells with high (220 ng/ml) TPA concentrations, which inactivates PKC, also restored CRE binding (Fig. 7C). The results of several CRE-binding assays are summarized in Table 1. CRE-binding (CREB) activity in Ats-aza cells at 33°C was generally more responsive to cAMP or sphingosine alone than to TTF1.

We note that the CREB complex is not thyroid specific, and, unlike TTF1, can be detected in Ats cells, the progenitors of the Ats-aza line (data not shown). The CREB factor of the Ats-aza cells is not AP1; binding was not affected by the addition of anti *c-fos/c-jun* antibodies (data not shown).

Assays of a CRE-chloramphenicol acetyltransferase (CAT) fusion transfected into Ats-aza cells confirmed

the results of the binding studies shown in Figure 7B, lanes 1 and 2. Cells cultured and transfected at 39°C showed more CAT activity than those at 33°C (data not shown).

Previously, we reported that the PKA activity of Ats-aza cells cultured at 33°C was reduced twofold compared with cells grown at 39°C (Avvedimento et al. 1991). These *in vitro* assays measured kinase activity after dissociation of PKA holoenzyme with cAMP. TPA-treated cells, however, retained high PKA levels. Furthermore, the reactivation of TTF1 and CREB by sphingosine and 8-Br-cAMP in Ats-aza cells at 33°C was not accompanied by an increase in overall PKA activity (data not shown). Nevertheless, loss of active TTF1 and CREB indicates that PKC lowers the effective concentration of PKA.

Nuclear migration of PKA catalytic subunit

The PKA catalytic subunit translocates to the nucleus in response to cAMP or to forskolin, which stimulates adenyl cyclase (Nigg et al. 1985a). We considered the possibility that v-Ras and PKC might inhibit the nuclear migration of the PKA subunit. Our enzyme assays did

Figure 6. TTF1 in sphingosine-treated cells is sensitive to phosphatase and reactivated *in vitro* by PKA. Shown is TTF1 binding in nuclear extracts of Ats-aza cells grown at 39°C (lane 1) or for 1 week at 33°C and then treated for 2 days with 10 μ M sphingosine and 1 mM 8-Br-cAMP (lane 2). The nuclear extract shown in lane 2 was treated with sweet potato acid phosphatase (lane 3). Phosphatase-treated extracts were incubated with activated PKC (lane 4), or with 50 units of PKA catalytic subunit for 10 min at room temperature (lane 5), or 50 units of PKA and 5 μ g of activated PKC (lane 6). The phosphorylating activity of the PKC preparation was verified by using histone 3S as substrate under incubation conditions used for the gel shift experiments (data not shown).

Figure 7. (A) Stimulation of PKC inhibits factor binding to a promoter containing a CRE. Shown is a gel retardation assay of an oligonucleotide containing the CRE region of the mouse *c-fos* promoter (5'-GTTCCGCCAGTGACGTAGGAAGTC-3') incubated with nuclear extracts (5 μ g of protein) from TL or Ats-aza cells cultured at 39°C (lanes 1 and 2, respectively). The Ats-aza cells were treated with TPA (12 ng/ml) for 1 day (lane 3), 3 days (lane 4), or 6 days (lane 5). The specificity of the retarded complex was established by competition with nonspecific oligonucleotides (data not shown). (B) Sphingosine restores CREB activity in cells exposed to v-Ras. Shown is CREB by nuclear extracts from Ats-aza cells cultured at 39°C (v-Ras⁻; lane 1) or 33°C (v-Ras⁺; lane 2). Ats-aza cells grown at 33°C for 1 week were treated for 2 days with 10 μ M sphingosine (lane 3), 1 mM 8-Br-cAMP and 10 μ M sphingosine (lane 4), or 1 mM 8-Br-cAMP (lane 5). In other experiments, CREB was fully restored by 1 mM 8-Br-cAMP treatment of Ats-aza cells cultured at 33°C. (C) TPA at high concentration suppresses v-Ras and restores CREB activity. CREB in Ats-aza cells at 39°C (lane 1), incubated for 1 week at 33°C (lane 2), or incubated for 1 week at 33°C and then treated for 12 hr at 33°C with 220 ng/ml of TPA.

not distinguish between nuclear and cytoplasmic PKA activity. Accordingly, we have used immunofluorescence and other assays to determine the cellular location of the PKA subunits under various culture conditions. These assays show a correlation between the activity of TTF1 (and CREB factor) and the nuclear concentration of the PKA catalytic subunit. Immunofluorescence assay indicates that the catalytic subunit entered the nucleus of Ats-aza cells cultured at 39°C after treatment with forskolin (Fig. 8A,B). In contrast, Ats-aza cells cultured at 33°C did not respond efficiently to forskolin; fewer nuclei were reactive, and the distinctive granular distribution of the anti-catalytic subunit antibody was absent (Fig. 8C,D). We conclude that v-Ras blocks the nuclear translocation of the catalytic subunit induced by cAMP. When the Ats-aza cells grown at 33°C were treated with sphingosine, nuclear transport of the subunit in response to forskolin was restored (Fig. 8E). The inhibition of subunit translocation by v-Ras therefore required active PKC.

Staining of Ats-aza cells with anti-regulatory subunit

antibodies (anti-RII) indicates that the RII subunit is concentrated in a cytoplasmic region very close to the nuclear membrane. The RII subunit did not diffuse through the cytoplasm after dissociation with forskolin and did not enter the nuclei (Fig. 8, cf. B and H).

The effects of PKC on the nuclear migration of the PKA catalytic subunit are also shown in Figure 9. PKA in nuclear fractions was estimated by following the phosphorylation of a PKA-specific peptide substrate (Fig. 9a). Kinase activity appeared in the nuclei of cells cultured at 39°C and treated with forskolin, cells cultured at 33°C, treated similarly, showed no increase in nuclear kinase (Fig. 9a, lanes 1–4). Stimulation of PKC with TPA (12 μ g/ml) prevented the transport of PKA in cells grown at 39°C, as shown by enzyme assay (Fig. 9a, lanes 5,6), by immunofluorescence (Fig. 8B), or by Western blotting of nuclear extracts (Fig. 9c).

Discussion

Thyroid-specific gene expression is maintained both in vivo and in cultured lines by cAMP. Expression of thyroid peroxidase, the sodium/iodide cotransporter, TSH receptor, and thyroglobulin is down-regulated in cells deprived of cAMP (Weiss et al. 1984; Saji et al. 1992). The effects of cAMP are mediated, at least in part, through the thyroid transcriptional factor TTF1. TTF1 is a phosphoprotein that is directly modified by PKA. When phosphorylated, it binds to and activates pTg. Dedifferentiation in starved thyroid cells is initially associated with the accumulation of nonmodified, inactive TTF1. With longer periods of starvation, TTF1 protein disappears and TTF1 gene transcription ceases (A.M. Musti, in prep.).

The dephosphorylation of TTF1 is the basis of dedifferentiation induced by the v-Ras oncogene in thyroid cells (this work; Avvedimento et al. 1991). In this paper, we demonstrate further that (1) v-Ras acts through PKC, and (2) PKC blocks the migration of the PKA catalytic subunit from the cytoplasm into the nucleus. Thus, the activity of nuclear *trans*-acting factors that are positively regulated by PKA is inhibited, and the transcription of their target genes is reduced.

v-Ras dedifferentiates thyroid cells

The rat thyroid cell line Ats-aza is transformed with a v-Ras temperature-sensitive mutant. Ats-aza cells dedifferentiate at the permissive temperature (33°C) and redifferentiate when returned to 39°C. Unmodified TTF1 is found in the nucleus of cells cultured at 33°C, suggesting that exposure to v-Ras reduces the activity of PKA. Consistent with this interpretation, we find that cAMP-dependent CREB activity and the expression of CRE-CAT fusions are also reduced in v-Ras-transformed cells. Similarly, the transcription of MGBCoA reductase, a thyroid housekeeping gene that is regulated by TSH and cAMP (Grieco et al. 1990), is also reversibly inhibited by v-Ras (M. Bifulco, pers. comm.).

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Figure 8. (See facing page for legend.)

PKC mediates the effects of v-Ras

Stimulation of PKC with the phorbol ester TPA, like exposure to v-Ras, induces thyroid cell dedifferentiation (this paper; Lombardi et al. 1988). Conversely, depletion of PKC by treatment with high levels of TPA, or inhibition of PKC with sphingosine, overcomes v-Ras and restores TTF1-binding and pTg activity. The reactivation of TTF1 is potentiated by suggesting that adenylyl cyclase is down-regulated in v-Ras-transformed cells, probably as a result of reduced TSH receptor concentrations (Berlingieri et al. 1990). CREB activity, in contrast to TTF1, is entirely restored by treatment of Ats-aza cells at 33°C. We believe that CREB proteins are preferred substrates for PKA and are phosphorylated more efficiently than TTF1 under conditions of limiting PKA activity.

These experiments implicating PKC as the mediator of v-Ras are supported by the direct demonstration of increased PKC activity in v-Ras-transformed cells (Spina et al. 1988). We have also observed a fivefold increase in cytosolic PKC when Ats-aza cells are shifted from 39°C to 33°C (A. Gallo, unpubl.).

Our evidence argues that PKC is an effector of v-Ras in thyroid cells. We note that in T lymphocytes, PKC activates c-Ras by inhibiting GTPase-activating protein (GAP) (Downward et al. 1990). v-Ras, whose activity is independent of GAP, could not be stimulated by PKC by this pathway.

PKC inhibits nuclear migration of the PKA catalytic subunit

These data indicate that v-Ras, or PKC, inhibits the accumulation of PKA catalytic subunit in the nuclei. Little is known about how the subunit enters the nucleus. It lacks a nuclear localization signal and is thought to translocate through diffusion. Nuclear translocation is rapid and reversible (Nigg et al. 1985a). The subunit appears to be anchored at discrete chromosomal sites, possibly in proximity to substrate transcriptional factors.

The down-regulation of nuclear PKA might also explain why v-Ras stimulates cell growth. PKA appears to suppress mitosis; inhibitors of PKA or transient decreases in levels induce maturation of *Xenopus* oocytes

(Maller and Krebs 1980). v-Ras also induces oocyte maturation (Kamata and Kung 1990; Pan and Cooper 1990; Sadler et al. 1990). Recent experiments indicate that coinjection of the PKA catalytic subunit with v-Ras prevents oocyte maturation and the accumulation of active maturation promoting factor (MPF) (S. Fisch, D. Grieco, M.E. Gottesman, and V.E. Avvedimento, in prep.; I. Dahr and G. VandeWoude, pers. comm.).

The catalytic subunit of PKA is bound to one of two regulatory subunits, RI or RII (see Taylor 1989). The two PKA holoenzymes have different affinities for cAMP (K_d RI = 1 μ M; K_d RII = 10 μ M) (Hofmann et al. 1975), different rates of turnover (RI $t_{1/2}$ = 31 hr; RII $t_{1/2}$ = 125 hr) (Weber and Hilz 1986), and distinct cytoplasmic locations. The RII complex is perinuclear, whereas the RI complex is broadly distributed in the cytoplasm (Nigg et al. 1985b; Meinkoth et al. 1990). Our preliminary evidence suggests that the source of nuclear catalytic subunit is the RII holoenzyme (V.E. Avvedimento and M.E. Gottesman, unpubl.). This is consistent with the absence of transcriptional response to cAMP of CRE-CREB-dependent genes in the PC12 mutant cell line A126-1B2. The concentration of RI PKA holoenzyme in this cell line is normal, whereas the RII holoenzyme is markedly deficient (Van Buskirk et al. 1985).

The linkage between transformation and nuclear PKA levels is not known. We do not know whether v-Ras/PKC blocks the dissociation of PKA holoenzyme or whether cytoplasmic or nuclear anchoring systems are affected. We can distinguish between two effects of v-Ras on PKA. One is rapidly reversible by treatment with a PKC inhibitor. A second effect is a change in the composition of PKA holoenzyme from an RII to an RI complex associated with cellular transformation (Schwartz and Rubin 1983; Cho-Chung 1990; Tortora et al. 1990). This change is expected to decrease nuclear PKA activity, thus inhibiting cAMP-dependent transcription and deregulating cell growth while retaining cytoplasmic and metabolic responses to cAMP.

Materials and methods*Cell lines*

The rat thyroid cell line TL (referred to previously as FRTL-5)

Figure 8. Immunofluorescence assays. Ats-aza cells were cultured at 33°C or 39°C and, where indicated, treated with 40 μ M forskolin for 40 min at 37°C. (A) Ats-aza cells grown at 39°C stained with anti-PKA catalytic subunit antibodies. The labeled subunit is concentrated in the region of the centrosome (see below) and is almost absent from the nucleus. (B) Ats-aza cells grown at 39°C as in A, after treatment with forskolin; staining is with anti-catalytic subunit antibodies. The label appears more diffuse in the cytoplasm and is present in the nucleus as discrete, bright spots. Treatment with dibutyryl or 8-Br-cAMP (10^{-4} M) reproduced the effects of forskolin (data not shown). At higher concentrations of cAMP, the labeled subunit appeared diffuse in both the cytoplasm and the nucleus (data not shown). (C) Ats-aza cells incubated at 33°C for 3 weeks were stained with anti-catalytic subunit antibodies. The stain distribution is essentially identical to A. (D) Ats-aza cells cultured at 33°C as in C, treated with forskolin. The cytoplasmic distribution of subunit is diffuse, as in B; however, discrete spots of label are absent from the nucleus. (E,F) As in D, except that the cells were preincubated at 33°C for 24 hr with 10 μ M sphingosine before forskolin treatment. Cells were double stained with anti-catalytic (E) and anti-tubulin (F) antibodies. Labeled granules are now evident in the nucleus (E). The anti-tubulin antibodies label the centrosome; the unstained nucleus is clearly defined. (G) Ats-aza cells at 39°C stained with anti-PKA regulatory subunit antibodies (anti-RII). The distribution of the RII subunit is perinuclear. (H) As in G, except that cells were treated with forskolin and stained with anti-RII antibodies. The RII subunit largely retains its perinuclear localization. Nuclear staining with Hoechst of Ats-aza cells grown at 39°C confirmed the nuclear localization of the catalytic subunit after forskolin treatment.

grows in chemically defined medium (Ambesi-Impimbatto et al. 1980). It was transformed with a Ki-MSV bearing a temperature-sensitive *v-ras* allele (Shih et al. 1970), yielding clone *Ats* (Colletta et al. 1983). *Ats* cells were transfected with a pTg-NEO fusion, and G418-resistant derivative clones (*Ats-aza*) were isolated after treatment with 5-azacytidine (Avvedimento et al. 1989).

DNA-binding assays

Assays for TTF1 binding used a 25-bp oligonucleotide [oligo(C)] derived from the rat pTg, which includes the high affinity site at -60 nucleotides relative to the transcription start site (Musti et al. 1987; Avvedimento et al. 1989; Civitareale et al. 1989). The sequence of this oligonucleotide is 5'-GCCACTGCCAGT-CAAGTGTTC-3'. The TTF1 core-binding site is underlined. The specificity of binding in some experiments was demonstrated by using a mutant oligonucleotide bearing a single point core mutation (CAAGTA•TTC). This mutation reduces the binding of TTF1 by >10-fold. Additional control experiments measured the binding of oligo(A), 5'-GATTACTCAAGTAT-TCTTAGCGGG-3', derived from the -150-nucleotide region of the rat thyroglobulin promoter. Oligo(A) binds a nonthyroid-specific nuclear factor (Civitareale et al. 1989).

DNA oligonucleotides were end-labeled with T4 polynucleotide kinase (Maniatis et al. 1982) and 50 fmol were incubated for 15 min at room temperature with 5 μ g of nuclear proteins (Wu 1985) in the presence of 80 μ g/ml of poly[d(I-C)], 5 mM spermidine in 10% glycerol, 10 mM HEPES (pH 7.9), 50 mM NaCl or 200 mM KCl, and 0.1 mM PMSF in a final volume of 25 μ l. After incubation, the reaction mixture was brought to 5 mM DTT. The DNA-protein complexes were resolved on 6% acrylamide gels in 0.5 $\times$ Tris-borate buffer (Fried and Crothers 1981).

Treatment of nuclear extracts with PKA or PKC was per-

formed at room temperature for 15 min in the binding buffer described above, with the addition of 1 mM ATP. Incubation of the extracts with sweet potato phosphatase was performed at room temperature with 0.5 units (sp. act. 600 U/mg, Sigma) for 5 min and stopped with 1 μ l of 2 mM Na vanadate and 1 μ l of 40 mM NaF. Rat brain PKC (a generous gift of Drs. I.B. Weinstein and M. Ueffing, Columbia University, New York) was activated with 2.5 μ g/ml of diolein and 5 μ g/ml of phosphatidylserine (Avanti Polar Lipids, Inc., Pelham, AL) as described previously (O'Brian et al. 1985), and PKA catalytic subunit (30.6 pmoles/ μ g, Sigma) or holoenzyme isolated from rabbit muscle or bovine heart was obtained from Sigma. Holoenzyme was activated with cAMP (final concentration, 0.5 mM), for 10 min at 30°C. The enzyme was stored frozen in 70 mM DTT. PKA or PKC was added to the binding mixture before the addition of spermidine and DNA (volume 16 μ l), and the binding reaction was initiated with the addition of these components. The enzymes used (phosphatase, PKA, or PKC) alone did not affect the migration or the intensity of free DNA.

RNA analysis

RNA was isolated from cells by the guanidium thiocyanate-acid phenol procedure (Chirgwin et al. 1979). Total RNA (20 μ g) was fractionated on 1% formaldehyde-agarose gels, transferred to nylon (Hybond), hybridized with the *Bgl*I-*Nco*I fragment of the neomycin resistance gene (Santerre et al. 1984), and washed as described (Maniatis et al. 1982).

Immunofluorescence

Cells were cultured on 1.2-cm-diam. glass coverslips to subconfluency in the presence of 5% serum without hormones. Where indicated, the cells were stimulated with 40 μ M forskolin or 10^{-4} M 8-Br-cAMP at 37°C for 40 min. The cells were then fixed

with 3.7% formaldehyde in PBS and reacted with rhodamine-tagged goat anti-rabbit (Miles Scientific) or fluorescein-tagged goat anti-mouse immunoglobulin (Jackson). After several washes in PBS, the coverslips were mounted on microscope slides with a 50% solution of glycerol in PBS. The specificity of anti-catalytic and anti-regulatory antibodies has been checked by immunoprecipitation. The percentage of positive nuclei with anti-catalytic antibodies was ~80%. The failure of ~20% of the cells to respond to forskolin may represent cell-to-cell heterogeneity.

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